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# Autophagy eliminates cytoplasmic β-catenin and NICD to promote the cardiac differentiation of P19CL6 cells

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ABSTRACT

Autophagy plays important roles in adipogenesis and neuron development. However, how autophagy contributes to cardiac development is not well understood. The main aim of our study was to determine the association between autophagy and myocardial differentiation and its roles in this process. Using a well-established in vitro cardiomyocyte differentiation system, P19CL6 cells, we found that autophagy occurred from the early stage of cardiac differentiation. Blocking autophagy by knocking-down of autophagy-related gene *Atg7* or *Atg5* inhibited the cardiac differentiation of P19CL6 cells. Further investigation demonstrated that LC3 and P62 could form a complex with β-catenin and NICD, respectively, and promoted the degradation of β-catenin and NICD. Enhancing autophagy promoted the formation of complex, whereas blocking autophagy attenuated the degradation of β-catenin and NICD. Taken together, autophagy could facilitate P19CL6 cells to complete the cardiac differentiation process through blocking Wnt and Notch signaling pathways.



## 1. Introduction

The heart is the first organ to be formed from mesodermal cells during embryogenesis, and all subsequent events in the life of the organism depend on its uninterrupted contractility. Heart development is an extraordinarily complex process involving the integration of multiple cell lineages into the 3D organ and its seamless connection to the vascular system [1], its developmental process is controlled by many signal pathways, such as Wnt, Notch, BMP and FGF [2]. Wnt and Notch signaling pathways play important roles in heart development, and emerging evidence has shown crosstalk between Wnt and Notch pathways [3].

Wnts are a family of secreted signaling proteins, which have a major influence on embryonic development, tumor progression, and stem cell differentiation. It has been demonstrated to have a biphasic role in cardiogenesis. At the early stage of cardiac development, it promotes cardiomyocyte differentiation, whereas at the later stage, Wnt signaling inhibits cardiogenesis [4]. That means Wnt signaling has differential stage-specific effects, functioning in either an agonistic or antagonistic fashion.

Notch signaling, another important pathway in cardiac differentiation, is thought to inhibit cardiac differentiation. ES cells deficient in the Notch downstream effector RBP-Jk generate more cardiomyocytes than wild-type counterparts, whereas ES cells expressing a constitutively active form of the Notch1 receptor display a reduced cardiac differentiation potential [5].

Although these studies collectively show that Notch signaling has inhibitory roles at a specific stage of the cardiac differentiation, some researchers demonstrate that Notch1 is required for cardiac progenitor cells differentiating into cardiomyocytes by positively regulating the expression of cardiac transcription factors in mice embryos and ES cells [6–10]. We previously investigated the interplay between Wnt and Notch signaling pathways during cardiomyogenesis in a pluripotent mouse embryonic carcinoma cell line (P19CL6). We found that Notch1 signaling interacts with Wnt/β-catenin pathway and plays a biphasic role at the early stage of cardiac differentiation [3]. But at the late differentiation stage, the component molecules of Wnt and Notch pathways are quitted from the differential process quickly. The mechanism is not clear.

It is well known that the degradation of intracellular proteins is involved in the regulation of a series of cellular processes, including cell-cycle division, DNA repair, cell growth and differentiation, apoptosis, etc. All proteins in eukaryotic cells are continually being degraded and replaced. Autophagy and the ubiquitin–proteasome system are two mechanisms for intracellular protein degradation [11]. Autophagy is mediated by lysosome, and is further divided into chaperone-mediated autophagy, microautophagy and macroautophagy. It is believed that *Atg5* and *Atg7* are essential genes for mammalian autophagy

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[12]. The ubiquitin–proteasome system is highly complex and mediated by ubiquitin, which participates in intracellular protein degradation in a specific manner [13].

It has been reported that the inhibition of the ubiquitin–proteasome pathway blocked the decrease of β-catenin and resulted in the accumulation of both β-catenin and plakoglobin in the nucleus [14]. Meanwhile, the substitution of the serine residues in the glycogen synthase kinase 3β (GSK3β) phosphorylation consensus motif of β-catenin inhibited ubiquitination and provided the first evidence that the ubiquitin–proteasome degradation pathway may act downstream of GSK3β in the regulation of β-catenin [15]. For Notch receptors, monoubiquitylation and endocytosis are prerequisites for efficient receptor activation and signaling [16]. The ubiquitylation of Notch receptor ligands is required for ligand endocytosis in the signal-sending cell and Notch signaling in the signal-receiving cell [17]. These literatures suggest that the intracellular protein degradation is essential for the cells.

Autophagy is also reported to be related with Wnt and Notch signaling pathways in different cell models. Autophagy negatively regulates Wnt signaling by promoting Disheveled (Dvl) degradation in the late stages of colon cancer development [18]. β-Catenin suppressed autophagosome formation and directly repressed the autophagy adaptor P62 expression [19]. In *Drosophila* oogenesis, the loss of autophagy leads to the activation of the Notch signal [20]. However, whether autophagy regulates Wnt and Notch signaling pathways in cardiac differentiation is not well understood.

In this study, we investigated the impact of autophagy during the cardiac differentiation process of P19CL6 cells, a well-established in vitro cardiomyocyte differentiation system (Fig. S1) [21]. We found that autophagy begins to occur at the early stage, and maintains at a high level during cardiomyocyte terminal differentiation. β-Catenin and NICD, as the effectors of Wnt and Notch signaling pathways, could form a complex with LC3 and P62 and be degraded by autophagy. As both Notch and Wnt pathways play inhibitory roles in the late stage of cardiac differentiation, the appropriate clearance of the components of two pathways could facilitate P19CL6 cells to complete the cardiac differentiation process.

## 2. Materials and methods

### 2.1. Cell culture

P19CL6 cell line was established in 1996 [22] and was used in this study. These cells are embryonic carcinoma cells that can be induced with 1% dimethyl sulfoxide (DMSO) into cardiomyocyte like beating cells. The induced cells express specific cardiac markers in a stage specific manner: NKX2.5, ISL1, α-cardiac myosin heavy chain (MHC), sarcomere myosin (MF20) and troponin T, troponin I, etc. This cell line has been used as a good model to study the mechanism of cardiac differentiation [21] since then. P19CL6 cells were cultured as described previously [23]. In brief, the cells were grown in a 60-mm tissue culture dish with growth medium containing α-minimal essential medium (Gibco BRL) supplemented with 10% fetal bovine serum (FBS, Hyclone USA), penicillin (100 U/ml) and streptomycin (100 mg/ml), and were maintained in a 5% $CO_2$ atmosphere at 37 °C. To induce cardiac differentiation, P19CL6 cells were plated at a density of $3.7 \times 10^5$ in a 60-mm tissue culture dish with the growth medium containing 1% dimethyl sulfoxide (DMSO). The medium was changed every 2 days. The days of differentiation were numbered consecutively beginning at the first day of the DMSO treatment (day 0).

### 2.2. Construction of Atg7 or Atg5 knockdown cell lines

Atg5/GV102RNAi construct (GCTTTACTCTCTATCAGGATGAGATTTC AAG AGAATCTCATCCTGATAGAGAGTAAAGC) and Atg7/GV102RNAi construct (CCAAGGTCAAAGGACAAAGATCTCGAGATCTTTGTCCTTTGACC TTGG) were synthesized by Shanghai GeneChem Co., Ltd. The transfection of the plasmids was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. To create stable cell lines, $2 \times 10^5$ P19CL6 cells were plated onto a 60-mm culture dish. When the cells reached approximately 50% confluence, P19CL6 cells were transfected with 5 μg Atg5 RNAi plasmid, Atg7 RNAi plasmid, and the scrRNAi plasmid using Lipofectamine 2000. G418 selection (1000 μg/ml) was continued for 7 days. The identification of stable cell lines was performed by examination for expression of *Atg7* and *Atg5*, respectively.

### 2.3. Luciferase reporter construction and luciferase assays

P19CL6 cells were plated into 24-well plates at $5 \times 10^4$ cells/well 24 h before transfection. TOP/FOP Flash, Hes1 luc, Hey1 luc plasmids (400 ng) and 20 ng control Renilla vector (phRLTK, Promega) were cotransfected with 2 μl transfection reagent (Lipo 2000). Lysates were collected 36 h after transfection, and luciferase activity was measured with the dual luciferase assay (Promega).

### 2.4. Western blotting analysis

Total protein extracts were obtained with lysis buffer (150 mM NaCl, 10 mM Tris [pH 7.2], 5 mM EDTA, 0.1% sodium dodecyl sulfate [SDS], 1% sodium deoxycholate, 1% Triton X-100) containing protease inhibitor cocktail (Sigma). Proteins were separated by electrophoresis on 8–15% SDS-polyacrylamide gels, transferred to nitrocellulose, and incubated with corresponding primary antibodies.

LC3, Atg5, P62 and Ub antibodies were purchased from MBL Co.; Atg7 antibody was purchased from CST Co.; β-catenin, NICD and GATA4 antibodies were purchased from Santa Cruz Co.; Troponin T antibodies were purchased from Abcam Co.; MF20 antibody was from Developmental Studies Hybridoma Bank, the University of Iowa.; α-actinin antibody was purchased from Sigma Co. The membrane was also probed for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. The blots were next incubated with peroxidase-conjugated IgG secondary antibody and developed using the enhanced chemiluminescence kit (Millipore).

### 2.5. Quantitative real-time RT-PCR

For mRNA analysis, total RNA was isolated with TRIzol reagent (Invitrogen), and 5 μg total RNA was reverse transcribed with random primers for cDNA synthesis in the presence of RNase inhibitor (Vigorous). The cDNA was used for quantitative RT-PCR with specific primers. Transcript levels were normalized to 18S rRNA level. The primers are listed in Table 1. Each value represents the average of at least three independent experiments.

**Table 1**
Primers used for quantitative real-time RT-PCR.

| Primers | Sequence (5′–3′) | Product size |
|---|---|---|
| *Isl1* | F: CTGCTTTTCAGCAACTGGTCA<br>R: TAGGACTGGCTACCATGCTGT | 123 |
| *Gata4* | F: CACCCCAATCTCGATATGTTTGA<br>R: GGTTGATGCCGTTCATCTTGT | 151 |
| α-*Mhc* | F: GCCCAGTACCTCCGAAAGTC<br>R: GCCTTAACATACTCCTCCTTGTC | 110 |
| β-*Mhc* | F: ACAACCCCTACGATTATGCGT<br>R: ACGTCAAAGGCACTATCCGTG | 100 |
| *Troponin T* | F: GGCAGAACCGCCTGGCTGAA<br>R: CTGCCACAGCTCCTTGGCCT | 109 |
| *Wnt3a* | F: TGGCTGAGGGTGTCAAAGC<br>R: CGTGTCACTGCGAAAGCTACT | 181 |
| *NICD* | F: CCCTTGCTCTGCCTAACGC<br>R: GGAGTCCTGGCATCGTTGG | 162 |

*2.6. Immunofluorescence staining*

For confocal microscopy, cells were cultured on confocal dishes for 24 h and then were fixed with 4% formaldehyde, permeabilized with phosphate-buffered saline containing 0.1% Triton X-100, and incubated with corresponding primary antibodies, which was followed by incubation with the secondary antibody conjugated to Alexa Fluor 488. Nuclei were stained with Hoechst 33342. The confocal dishes were examined with an Olympus confocal microscope (Olympus Corporation, Tokyo, Japan).

*2.7. Co-immunoprecipitation assay*

Co-immunoprecipitations were performed according to the manufacturer's instructions (Roche, Basel, Switzerland). The lysates isolated from P19CL6 cells were incubated with specific antibody or normal IgG for overnight at 4 °C with gentle rotation, and then with 50 μl of protein G-Agarose (Roche) at 4 °C for 4 h with gentle rotation. Immunoprecipitates were washed three times with wash buffer and subjected to SDS-PAGE electrophoresis and were then detected with specific antibodies.

*2.8. Statistical analysis*

The data are displayed as mean ± standard deviation (SD). Comparisons were analyzed by Student's *t*-test or ANOVA. Significance was analyzed using the SPSS10.0 software and a *P*-value $<0.05$ was considered to indicate statistical significance.

## 3. Results

*3.1. Autophagy occurs in the early stage of cardiac differentiation of P19CL6 cells*

To determine whether autophagy is involved in the process of P19CL6 differentiation, we firstly examined LC3 activation during the whole stage of P19CL6 differentiation. The LC3 activation level was maintained at the low level before day 3 of the induction, but it rapidly increased

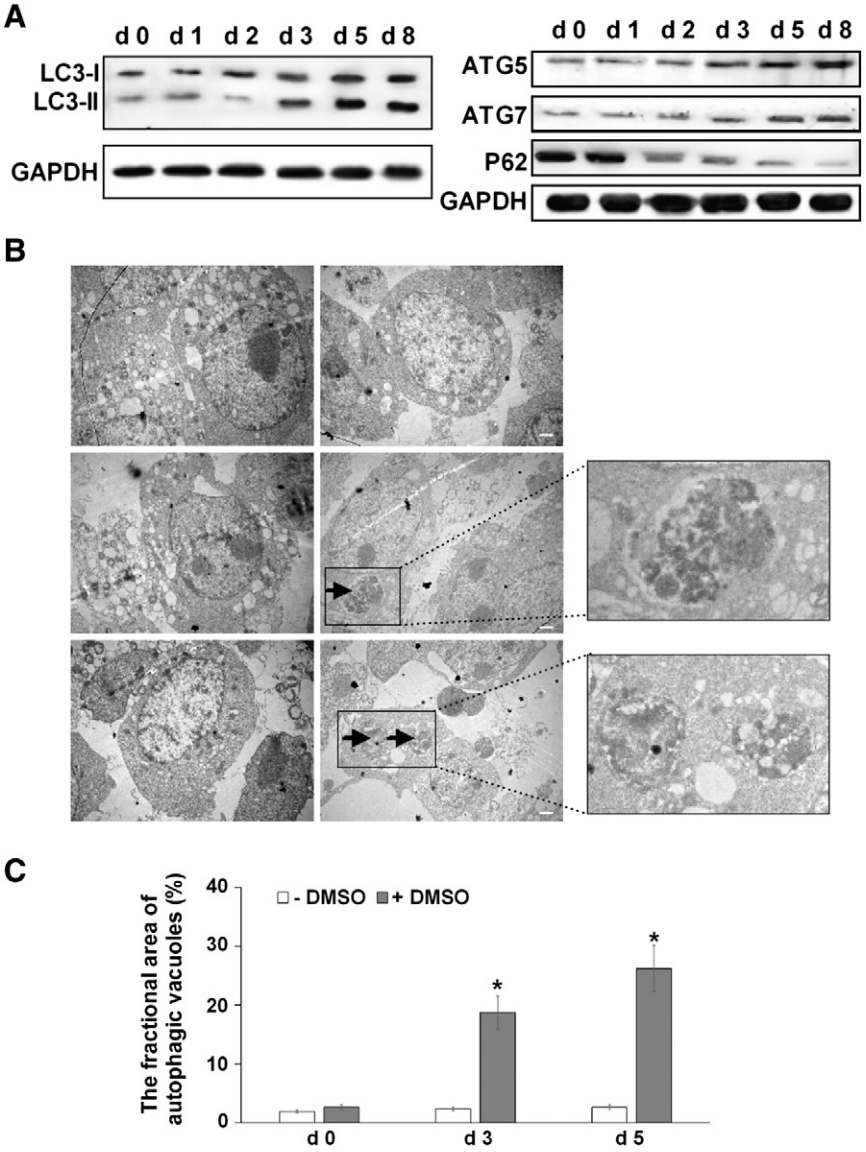


**Fig. 1.** Autophagy occurs in the early stage of cardiac differentiation of P19CL6 cells. (A) Autophagy was analyzed in DMSO-induced P19CL6 cells by Western blot. The expression of LC3, Atg5, Atg7 and P62 at the indicated time-points was determined, and GAPDH was used as an internal control. (B) Autophagic vacuoles (arrowheads) were observed at day 3 in DMSO-induced P19CL6 cells under an electron microscope (bar = 1 μm). (C) The percentage of fractional area of autophagic vacuoles was calculated using Image-Pro Plus and compared with the control group at the indicated time-points. P19CL6 cells without DMSO induction were used as the control. n = 10, each bar represents mean ± S.D. $^*P < 0.05$ vs. the control at the same time-points.

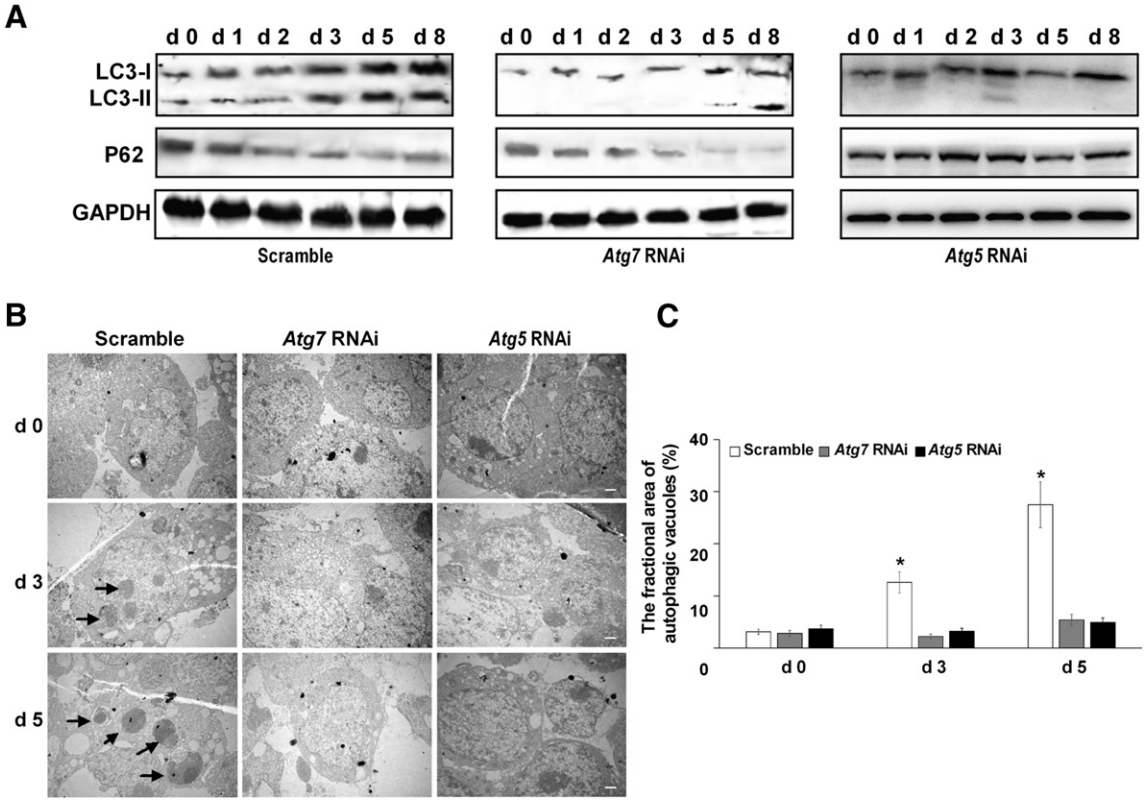


**Fig. 2.** Interfering *Atg7* or *Atg5* attenuates autophagy occurrence during the cardiac differentiation. P19CL6 cells were treated with siRNA against *Atg7* (Atg7 RNAi), *Atg5* (Atg5 RNAi), or scramble RNA (Scramble) as the control, and then the following examinations were performed. (A) The activation of LC3 and expression of P62 were analyzed with Western blot at the indicated time-points. GAPDH was used as an internal control. (B) Electron microscopy was used to detect the autophagic vacuoles. The autophagic vacuoles are indicated by arrowheads (bar = 1 μm). (C) The percentage of fractional area of autophagic vacuoles was calculated using Image-Pro Plus. The Scramble group was used as the control. n = 10, each bar represents mean ± S.D. $^*P < 0.05$ compared with the Scramble group.

from day 3 and remained at a high level until day 8. The expression of two autophagy related genes, Atg5 and Atg7, also increased significantly from day 3, and on the contrary, P62 expression was reduced (Fig. 1A).

In order to observe the formation of autophagic vacuoles intuitively, the electron microscopy was employed. Without DMSO induction, the autophagic vacuoles in P19CL6 cells are hard to observe. On the contrary, the autophagic vacuoles were observed from day 3 in P19CL6 cells treated with DMSO, and the fractional area of autophagic vacuoles compared with the whole cell area was increased gradually along with the days of differentiation (Fig. 1B, C). These data confirm that autophagy indeed occurs from the early stage of differentiation, and maintained a high level at the late stage.

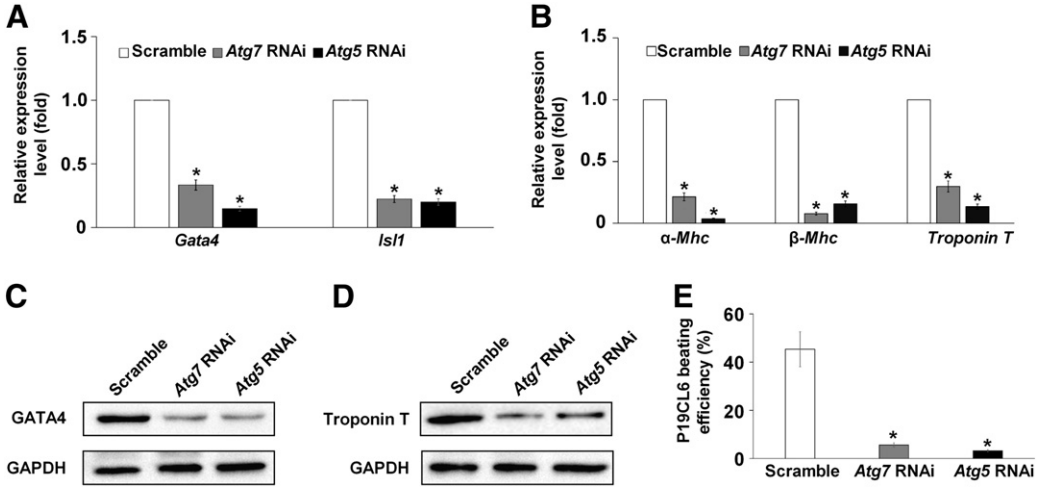


**Fig. 3.** Interfering autophagy blocks the cardiac differentiation. The expression of cardiac specific markers was analyzed by qRT-PCR (A, B) and Western blot (C, D) at day 4 for the early stage markers (*Gata4* and *Isl1*) or at day 12 for the later stage markers (α-*Mhc*, β-*Mhc* and troponin T) in P19CL6 cells treated with siRNA against *Atg7*, *Atg5*, or scramble RNA. (E) The cardiac differentiation efficiency was calculated by counting the beating cells over the total cell numbers at day 16 of induction. Each bar represents mean ± S.D. $^*P < 0.05$ compared with the Scramble group.

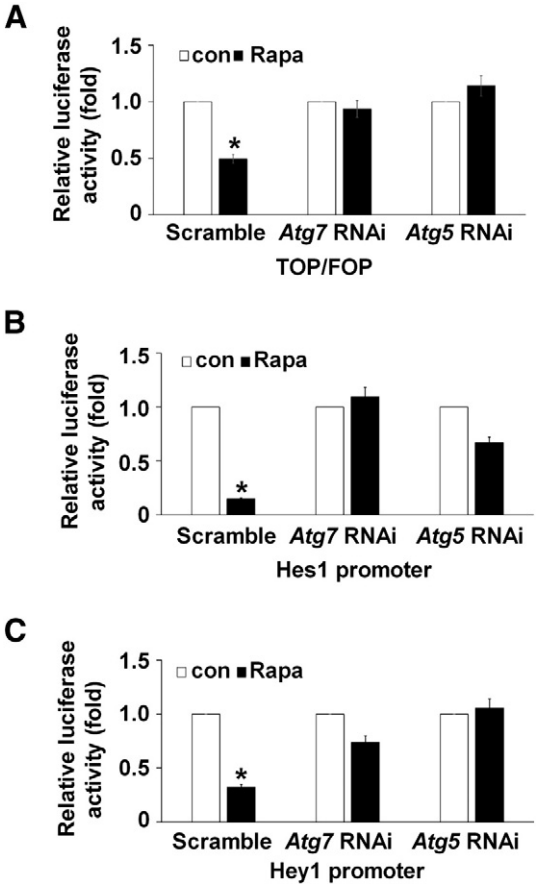


**Fig. 4.** Autophagy influences Wnt and Notch signaling pathways. Luciferase reporter assays were performed by transfecting TOP/FOP Flash (A), Hes1 (B) or Hey1 (C) luciferase reporter in the cells treated with or without rapamycin (Rapa, 200 nM, Sigma), an agonist of autophagy, for 24 h. The cells treated with placebo were used as the control. Data are shown as fold activation over that of the control with means + S.D. (n = 3). $^*P < 0.05$ vs. the control.

### 3.2. Interfering Atg7 or Atg5 attenuates autophagy occurrence during the cardiac differentiation

To investigate whether the induction of autophagy has any effect on P19CL6 differentiation, we established three cell lines: a cell line that was stably knocked-down *Atg7* (Atg7 RNAi), a cell line that was stably knocked-down *Atg5* (Atg5 RNAi), and a control cell line that was transfected with scramble nucleotides (Scramble) (Fig. S2).

Western blot analysis indicated that when Atg7 or Atg5 was knocked-down, LC3-II activation level was significantly reduced during the whole process of differentiation. Meanwhile, the expression of P62 was maintained in similar level as the Scramble group in *Atg7* RNAi group or sustained no change in *Atg5* RNAi group (Fig. 2A). Electron microscopy also showed that autophagy was significantly inhibited when *Atg7* and *Atg5* were knocked-down (Fig. 2B, C), suggesting that Atg7 and Atg5, as in other physiological processes, play roles in the autophagy occurrence during the cardiac differentiation.

### 3.3. Interfering autophagy blocks the cardiac differentiation

Considering that Atg7 RNAi and Atg5 RNAi cell lines displayed lower autophagy occurrence rate compared with the scramble cells, further investigation was carried out to explore whether the two genes are correlated with cardiac differentiation. Real-time RT-PCR analysis demonstrated that compared to scramble cells, when we knocked-down *Atg7* or *Atg5* expression, the expression of some cardiac specific markers, including *Gata4* and *Isl1* (the early-stage markers, Fig. 3A) and *Troponin T*, α-*Mhc* (α myosin heavy chain) and β-*Mhc* (β myosin heavy chain) (the late-stage markers, Fig. 3B), was notably decreased in the *Atg7* or *Atg5* knocked-down cells. Western blot assay also indicated that knocking-down of Atg7 or Atg5 influences the expression of GATA4 (Fig. 3C) and Troponin T (Fig. 3D).

As the most intuitive cell morphology, the P19CL6 beating efficiency was calculated, and the results showed that when autophagy was blocked by *Atg7* or *Atg5* RNAi, the counting of P19CL6 beating cells was remarkably lower than those of the control cells (Fig. 3E). The results suggest that endogenous autophagy occurrence is correlated with cardiac differentiation.

### 3.4. Autophagy influences Wnt and Notch signaling pathways during the cardiac differentiation

We have demonstrated that autophagy is essential for cardiac differentiation. Next, we investigated how autophagy affects the cardiac differentiation. It has been reported that autophagy can interfere Wnt and Notch signaling pathways in cancer development and *Drosophila* oogenesis. Therefore, we examined whether Wnt and Notch signaling pathways can be regulated by autophagy during the cardiac differentiation of P19CL6 cells. TOP/FOP-Flash reporter assay demonstrated that the application of rapamycin, an autophagy agonist, resulted in decreased TOP/FOP Flash luciferase activity of the reporter. When *Atg7* or *Atg5* was knocked-down, the addition of rapamycin failed to affect TOP/FOP Flash luciferase activity of the reporter in P19CL6 cells (Fig. 4A).

The influence of autophagy on Notch signaling pathway was consistent with the data obtained on Wnt signaling pathway. The addition of rapamycin resulted in decreased luciferase activity of the reporters that harbor *Hes1* promoter (Fig. 4B) or *Hey1* promoter (Fig. 4C), the direct downstream targets of Notch signaling pathway. Meanwhile, the knock-down of *Atg7* or *Atg5* blocked the rapamycin effect (Fig. 4B, C).

These data demonstrate that the classical Wnt/β-catenin and Notch pathways can be regulated by autophagy during the cardiac differentiation, which led us to explore the detailed molecular mechanism.

### 3.5. Autophagy could promote the degradation of β-catenin and NICD

It has been reported that autophagy can negatively regulate Wnt and Notch signaling pathways but the mechanism is not well understood. During the cardiac differentiation process of P19CL6 cells, the expression of *Wnt3a* (Fig. S3A), *Notch1* (Fig. S3B), β-catenin and NICD1 (Fig. S3C) exhibited a specific expression pattern, indicating that Wnt and Notch signaling pathways were activated at the early stage of cardiac differentiation. Immunofluorescence staining demonstrated that LC3 is located in β-catenin and NICD at day 4 of induction (Fig. 5A), suggesting that LC3 might form a complex with β-catenin and NICD. To verify this hypothesis, co-IP assay was employed at day 4 of differentiation, the results showed that LC3 formed a complex with β-catenin (Fig. 5B) and NICD (Fig. 5C), respectively. Co-IP assay demonstrated that P62 was also contained in the complex with β-catenin (Fig. 5D) and NICD (Fig. 5E). As we know, P62 is an important adaptor in selective autophagy [24]. It recognizes Ub substrates and transports these ubiquitinated proteins into larger aggregates for autophagic degradation. So we detected the status of ubiquitination by Western blot assay. The results showed that ubiquitination mainly occurred in small molecular weight proteins at day 0 and occurred in large molecular weight proteins at day 4 or day 8 (Fig. S4A). Co-IP assay results also demonstrated that ubiquitinated NICD or β-catenin at day 4 was more than those at day 0 (Fig. S4B).

We also tested whether autophagy activation could increase the interaction of LC3 with β-catenin or NICD. Immunofluorescence assay showed that when the autophagy was activated by rapamycin at day 0 of differentiation, the formation of complex which includes LC3 and β-catenin (Fig. 5F) or NICD (Fig. 5G) was significantly induced.

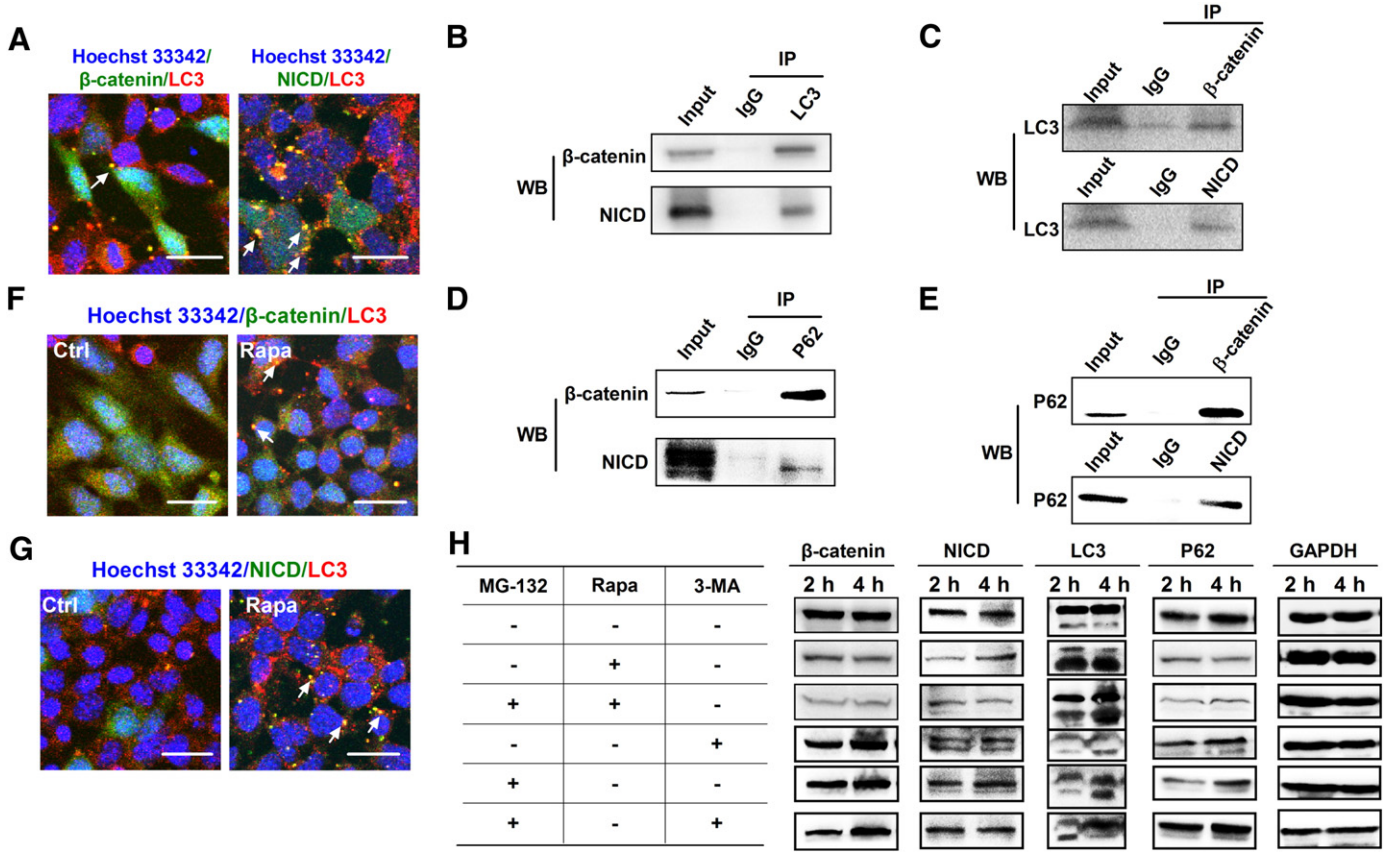


**Fig. 5.** Autophagy promotes the degradation of β-catenin and NICD. (A) Immunofluorescence staining was performed in P19CL6 cells induced by DMSO for 4 days. Endogenous expression and distribution of β-catenin and LC3 or NICD and LC3 were observed under the confocal microscope. The colocation of β-catenin and LC3 or NICD and LC3 is indicated by arrowheads (bar = 20 μm). (B) The cell lysates of P19CL6 cells induced to day 4 were coprecipitated with anti-LC3 antibody or normal IgG as a control antibody, and then detected by anti-β-catenin or anti-NICD antibody, 10% of total cell lysates were used as input. (C) The cell lysates of P19CL6 cells induced to day 4 were coprecipitated with anti-β-catenin and anti-NICD antibody, or normal IgG as a control antibody, and then detected by anti-LC3 antibody, 10% of total cell lysates were used as input. (D) The cell lysates of P19CL6 cells induced to day 4 were coprecipitated with anti-P62 antibody or IgG as a control antibody, and then detected by anti-β-catenin or anti-NICD antibody, 10% of total cell lysates were used as input. (E) The cell lysates of P19CL6 cells induced to day 4 were coprecipitated with anti-β-catenin and anti-NICD antibody, or normal IgG as a control antibody, and then detected by anti-P62 antibody, 10% of total cell lysates were used as input. (F, G) Immunofluorescence staining was performed in P19CL6 cells treated with or without rapamycin (Rapa, 200 nM). Endogenous expression and distribution of β-catenin and LC3 or NICD and LC3 were observed (bar = 20 μm). (H) P19CL6 cells were induced by DMSO to day 4, and then treated with rapamycin (Rapa, 200 nM), 3-methyladenine (3-MA, 10 mM, Sigma) or MG-132 (10 μM, Sigma), respectively. Total protein was isolated for Western blot analysis to detect the endogenous expression of β-catenin, NICD, LC3 or P62. GAPDH was used as an internal control.

As mentioned above, one of the functions of autophagy is to degrade the redundant cellular proteins. Wnt and Notch signaling pathways must be turned down at a certain stage of cardiac differentiation. It is interesting to know whether autophagy mediates the degradation of some component molecules in these two signaling pathways. Western blot results demonstrated that the level of β-catenin and NICD was significantly decreased in the cells treated with rapamycin [25], an autophagy agonists, along with enhanced LC3 activation and decreased P62 expression. While when we treated the cells with 3-MA [26], an autophagy inhibitor, the autophagy occurrence was blocked and the expression of β-catenin and NICD was increased (Fig. 5H). In order to exclude the effect of proteasome pathway, the cells were treated with proteasome inhibitor MG-132 [27]. There was almost no change on the expression of β-catenin and NICD, as well as LC3 and P62, compared with the control cells treated with placebo. This indicates that autophagy could influence the Wnt and Notch signaling pathways via accelerating the degradation of β-catenin and NICD.

## 4. Discussion

The main aim of our study was to determine the association between autophagy and differentiation related signaling pathways and its role during the process of cardiomyocyte differentiation.

It was well known that autophagy takes part in adipogenesis and neuron development. However, how autophagy contributes to development is not well understood, although it has been proposed that autophagy may influence cell differentiation either by impairing new protein synthesis or new organelle formation or by accelerating turnover of redundant proteins or organelles. Atg7 plays an important role in normal adipogenesis by inferring autophagy and the disruption of *Atg7* gene has a unique anti-obesity and insulin sensitization effect [28]. Ambra1 is a positive regulator of the Beclin1-dependent program of autophagy and its functional deficiency in mouse embryos leads to severe neural tube defects associated with autophagy impairment, accumulation of ubiquitinated proteins, unbalanced cell proliferation and excessive apoptotic cell death [29].

As we know that autophagy plays important roles in development [30], there are few reports in the field of myocardial differentiation. Inhibiting FRS2-α mediated signals increases autophagy and promotes myocardial differentiation [31].

In the heart, FIP200 (focal adhesion kinase (FAK) family kinase-interacting protein of 200 kDa, also known as RB1CC1) is a ULK1 (an Atg1 homologue)-interacting protein and has a molecular function similar to that of yeast Atg17, although it shows no sequence homology with any yeast Atg proteins [32]. FIP200 −/− mice are also embryonically lethal between E13.5 and E16.5 as a result of defective heart and liver development [33].

All these literatures have demonstrated that autophagy plays important roles in differentiation or development. As autophagy usually affects its functions by eliminating protein which should be degraded, how autophagy influences differentiation is to be considered. Wnt, activin/Nodal, BMP, FGF and Notch signaling pathways have been investigated extensively for their roles during cardiogenesis. We previously documented a specific reciprocal interaction between Wnt and Notch1 signaling pathways during the early stage of cardiac differentiation of P19CL6 cells [3].

Wnt/β-catenin signaling pathway is centered on β-catenin. In the absence of canonical Wnt signaling, β-catenin complex with APC and AXIN is phosphorylated by glycogen synthase kinase 3β (GSK3β) in a degradation box, which is polyubiquitinated for proteasomal degradation [34]. In the presence of Wnt ligands, binding to its receptor complex, GSK3β is inhibited, leading to the stabilization of cytoplasmic β-catenin. Accumulated cytoplasmic β-catenin subsequently translocates to the nucleus and initiates its target genes' transcription through T-cell factor (TCF)–lymphoid enhancer factor (LEF) transcription factors [35,36].

Notch signaling plays important roles in determining cell fate during development and is activated upon cell-to-cell contact as a result of interactions between Notch receptors and their ligands (Delta or Jagged). Ligand binding results in proteolytic cleavage of Notch receptors by γ-secretases, leading to the release of a 80-kDa intracellular domain of Notch (NICD), which then translocates to the nucleus and complexes with the transcription factor recombination signal sequence binding protein-Jkappa (RBP-Jk) to activate the transcription of its downstream target genes [37].

Through our study, we found that Wnt and Notch signaling pathways were both activated at the early stage of differentiation, suggesting that the two signaling pathways could promote cardiac differentiation, but at the late stage, both signaling pathways were closed, in which many mechanisms might be involved. As one of the protein elimination pathway, whether autophagy played important roles in degrading the long-lived proteins, such as β-catenin and NICD, is still unknown. Our results indicate that autophagy could influence the Wnt and Notch signaling pathways via accelerating the degradation of β-catenin and NICD.

## 5. Conclusion

Our results provide evidence for the function of autophagy in cardiac differentiation. When autophagy is inhibited, the cardiac differentiation is reduced. The main effectors of Wnt and Notch signaling pathways, β-catenin and NICD, could form a complex with LC3 and P62 and could then be degraded by autophagy. As both Notch and Wnt pathways play inhibitory roles in the late stage of cardiac differentiation, autophagy could facilitate P19CL6 cells to complete the cardiac differentiation process through inhibiting Wnt and Notch signaling pathways.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cellsig.2014.07.028.

## Declaration

The funders had no roles in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Competing interests: the authors declare no conflict of interest.

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